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Our group has developed a model immunotherapy system using a chimeric T cell receptors to redirect cytotoxic T cells to tumors. The efficacy of this approach in reducing tumor burden has been demonstrated using our constructs in models of ovarian carcinoma. To apply this technology to prostate tumors, we will alter the specificity of patient-derived lymphocytes through stable modification with chimeric receptor genes consisting of a targeting molecule linked to a T cell activation molecule (the γ subunit common to the Fc receptors, CD28 co-stimulatory receptor or Syk kinase). We have cloned, expressed, and immunized mice with two antigens expressed specifically on a large proportion of prostate tumors. These immunized mice will be used to produce prostate-specific monoclonal antibodies from which the scFv will be prepared. In addition, we have produced retroviruses capable of transducing human PBL with a chimeric receptor gene. Three targeting molecules have been used. We demonstrated their efficacy and the ability of the transduced lymphocytes to kill a prostate carcinoma line, LNCaP. We have started to put these constructs into lentiviral vectors which have the ability to transduce non-replicating cells. This therapeutic strategy may allow new approaches towards the adoptive immunotherapy of prostate cancer in humans.

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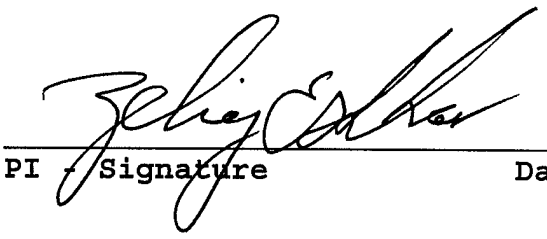

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Table of Contents

Introduction

The immunotherapy of cancer and viral diseases has several advantages over the classical therapies of chemotherapy and radiation. Immunotherapy is expected to be more specific for diseased tissue, and less toxic to the healthy organs of the patient. Nonetheless, in most cancer cases the immune system of the patient fails to cure the disease. To overcome this problem and to enhance the ability of the patient's own immune cells to fight fatal diseases, our group has pioneered the 'T-body' approach. In this approach, patient-derived effector cells (T or natural killer (NK) cells) are transduced with gene encoding a chimeric receptor comprised of a ligand or antibody-derived variable regions specific for a tumor antigen and linked to an effector cell activation molecule, such as the Fc receptor gamma (γ) chain. The transduced effector cells are thereby redirected to tumor cells, and upon reintroduction to the patient, are expected to kill target cells expressing the selected antigen. This approach combines the ability of T cells to home to, penetrate, and eliminate large solid tumors with the ability of antibodies to recognize a pre-selected antigen with high specificity and affinity without MHC restriction

The overall objective of the project is to refine and optimize the T-body approach and further develop it towards cell mediated immunotherapy of prostate cancer. Specifically, we are optimizing the composition of the chimeric receptor, directing it towards prostate cancer antigen by the generation of new scFv against prostate cancer surface antigens and improve the means of introducing such chimeric receptors into human T cells.

Body

The goal of this project is to retarget T cells to prostate tumors. This retargeting will be done by placing a single chain Fv fragment (scFv) onto a T cell. A crucial step is to find suitable antibodies. In the first year we have been concentrating on two antigens which are expressed on the surface of prostate cells, prostate specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA). The DNA sequence encoding each of these proteins was isolated from LNCaP cells by RT-PCR. To express protein to use for an antigen these sequence were cloned into mammalian and bacterial expression vectors.

Cloning and Expression of PSMA

PSMA was found as the antigen for the antibody 7E11 (1, 2). It is a type II membrane protein so its carboxyl terminus is extracellular with a transmembrane domain and a short intracellular domain. It is made up of 750 amino acids giving it a molecular weight of 84 kDa before glycosylation. There are 9 potential glycosylation sites. In mammalian cells it has a molecular weight of about 100 kDa. It has an enzymatic activity of a glutamate carboxypeptidase (3) (Figure 1).

RNA was isolated from the LNCaP human prostate cancer cell line using the Ultraspec™RNA isolation system (Biotecx Laboratories, Houston, TX). Reverse transcription was done using a Reverse Transcription System (Promega, Madison, Wisconsin) with 0.5 μg of oligo(dT) as primer for a 20 μl reaction mixture. The resulting cDNA was used as template to PCR the coding sequence of PSMA in two pieces and PSCA. The primers have restriction sites to clone the PCR product into pcDNA3 (Table 1).

Specifically the nucleotide sequence used was from Genbank (gb:humpsm). The protein sequence is in Swissprot (sw:psm_human). The 5' PCR primer (#26641) started 8 nucleotides before the initiating ATG. Before that sequence a Hind III site (relevant

restriction sites are underlined) was added to facilitate cloning. The nucleotide sequence representing the amino terminal fragment of PSMA was isolated as a PCR fragment down to the pre-existing EcoR I site (nucleotides 1572-1577) in the PSMA sequence (amino acids 437-439). When cloned into pcDNA3 this gave plasmid #342. The carboxyl terminal fragment was cloned by using for PCR the primer #26643 from the pre-existing EcoR I site mentioned above and primer #26644 to the carboxyl terminus of the protein (amino acid 750) where a BamH I site was added to facilitate cloning. When cloned into pcDNA3 this gave plasmid #344.

Each fragment was excised and cloned into pcDNA3-HA to give plasmid #350 with the entire PSMA molecule fused to an epitope tag from influenza hemagglutinin (HA-tag). This plasmid has the PSMA-HA coding sequence with a CMV promoter and a bovine growth hormone poly(A) site (Figure 2). It has the bacterial neo gene for selection by G418 in mammalian cells and an amp gene for selection with ampicillin in bacteria. This plasmid was transfected into NS0 cells and no G418 resistant transfectants were obtained.

For expression in *E. coli* fragments of the PSMA coding sequence were cloned into pGEX-3X to produce fusions to glutathione-S-transferase (GST). These fusions are expressed from an inducible hybrid trp-lac promoter. The plasmid contains the lacI gene to repress the lac promoter until induction by IPTG. The PSMA clone was used as a template in a PCR reaction using primers #27909 and #26644 to give a fragment coding from amino acid 274 to amino acid 439 (166 aa) which is in plasmid #371 (Figure 1). This protein fragment is from the amino terminus of the carboxypeptidase domain, which is now fused to the 218 amino terminal amino acids of glutathione-S-transferase to give a protein about 42 kDa (Figure 3).

A fragment from the carboxyl terminal end was also expressed in pGEX. Primer #29076 gave a PCR fragment, which coded for a protein segment starting from amino

acid 599, just after the last cysteine so that the protein fragment would not have a cysteine in it. Primer #26644 coded to last amino acid, number 750. When cloned into pGEX it produced plasmid #380 (Figure 1). This fragment is 152 amino acids and should give a fusion protein of about 40 kDa.

When transformed into bacteria TG-1 and induction by IPTG both plasmids produced proteins of the appropriate sizes in insoluble inclusion bodies.

Cloning and Expression of PSCA

Prostate stem cell antigen (PSCA) is a GPI-linked, Ly6-like protein. The mature protein has 79 amino acids but with 4 N-glycosylation site, it has a molecular weight of 24 kDa. It has 4 disulfide bonds like CD59 and cobra toxins. The sequence used to clone it came from Genbank (gb:af043498). PSCA was cloned using primers #26586 and #26587. The 5' primer, #26586, included a Hind III site and 11 nucleotides before the initiating ATG codon. The 3' primer, #26587, placed a Xho I site after the termination codon. When this PCR fragment was cloned into similarly cut Bam HI-Xho I pcDNA3 it resulted in plasmid #340. Our clone has a T at position 329 instead of a C. This is a silent change as in both cases it codes for alanine in the GPI signal. This was cloned into pcDNA3 (Figure 4).

For expression of the unmodified protein without the GPI linkage but rather as a transmembrane protein in mammalian cells this plasmid was used as the template in a PCR reaction using primers #27190 and # 27191. Primer #27190 placed a Xba I site before the amino acid sequence LQPG that is the amino terminus of the mature protein. Primer #27191 placed a Bst EII site after the NASGAHA sequence near the carboxyl terminus of the PSCA protein thereby removing the hydrophobic domain signaling GPI addition. This fragment was cloned into a RSV-gamma expression vector to give plasmid #353. In this vector, the mature PSCA protein has a mouse immunoglobulin light chain

leader and a human Igε gamma chain transmembrane region. Between the PSCA and the membrane is a YOL epitope tag and an IgG hinge spacer (Figure 5).

A vector to express PSCA with a YOL epitope tag was produced. The carboxyl terminus of the tag was placed on with primer #27237. The amino terminus of the tag was placed on with primer #27186. The PSCA with the YOL tag was cloned into the pcDNA3 vector (Figure 6).

PSCA was expressed as a fusion to IgG constant region sequences to produce a PSCA-IgG fusion protein. For expression as a PSCA-IgG chimeric protein, primers #26586 at the extreme amino terminus of the protein and # 27456 were used. Primer #27456 places a Bam HI site before the hydrophobic GPI signal at the carboxyl end of the PSCA protein. This enables the fusion to the IgG constant region in the expression vector driven by the CMV promoter (Figure 7). This plasmid (#360) was used to transfect by means of calcium phosphate 293T cells, human embryonic kidney cells that have been transformed with adenovirus and express SV40 T antigen. After 7 hours the medium was collected. This protein was purified on a Protein A column and used to immunize mice.

For bacterial expression of PSCA, its coding sequence was cloned into pGEX-3X using primers 28254 and 27748. This places a Bam HI restriction site in front of six amino acids before the beginning of the mature PSCA sequence and a EcoR I site at the end of the coding sequence for the mature PSCA protein enabling insertion into the pGEX-3X vector and production of a GST-PSCA fusion protein. These 85 amino acids fused to the GST portion gives a protein of a molecular weight of 33 kDa. Upon induction with IPTG the fusion protein is expressed and found in the insoluble inclusion body fraction.

Immunization of mice for monoclonal antibody production

Mice were immunized with each of the recombinant proteins described above using standard protocols. To evaluate the specificity and titer of antibodies produced following such immunizations we employed two assays: immunofluorescence staining of viable cells, and ELISA, using glutaraldehyde-fixed cells. FACS was used to measure the ability of antibodies in the mouse sera to stain LNCaP cells. Serum from mouse PSCA#5 stained LNCaP nicely at a dilution of 1:450 whereas normal mouse serum only stained at a dilution of 1:50. SKBr3 cells, a human mammary epithelial cell line which do not express PSCA or PSMA, do not stain.

Serum from mouse PSMAII3 stained LNCaP nicely at a dilution of 1:1350, whereas it stained SKBr3 at a dilution of 1:50.

These sera were tested in a whole cell ELISA. An ELISA plate was coated with a solution of 2 mg/ml of poly-lysine. Then LNCaP cells were allowed to bind. After washing with PBS glutaraldehyde (0.25%) was added for 15 minutes at 37°C. After rinsing with PBS the plates were blocked with 1% BSA. Dilutions of serum were added for 1 hour at 37°C, the plates were washed and developed with peroxidase-conjugated goat anti-mouse antibody. Both sera reacted with LNCaP as the PSCA serum and the PSMA serum reacted more than normal mouse serum (Figure 8).

These mice serve now as the source of immune cells to generate hybridomas and monoclonal antibodies.

Retroviral Constructs

Moloney based

The BULLET system of Bolhuis and colleagues was attempted first (4). This system involves the transient transfection of three plasmids to make a transducing retrovirus. One plasmid codes for the envelope protein, which could be from an ecotropic

virus, an amphotropic virus, or from the gibbon ape leukemia virus (GaLV). Another plasmid supplies the gag and pol proteins. The transfer vector with the LTRs and packaging signal carries an insert with the gene of interest (Figure 9). In our experiments this insert was specially designed. The scFv used in our initial experiments was from an antibody with specificity to trinitrophenol (TNP). This facilitates the testing of the genetically modified lymphocytes to kill a wide variety of cells as any cell can be made a target simply by treating with picryl chloride to label the cell surface with the trinitrophenyl (TNP) group. Another advantage of this scFv is that we have available an anti-idiotypic monoclonal antibody for detecting by FACS expression of this scFv on the cell surface. Genetically fused to this scFv is the co-stimulatory molecule CD28 to enable the production of a chimeric protein. This provides spacing of the scFv from the cell surface and permits the formation of heterodimers with endogenous CD28 molecules. The CD28 molecule has previously been used in chimeric receptor constructs (5, 6). In addition, CD28 signaling has been shown to prevent apoptosis of the lymphocyte. Attached to the carboxyl terminus is the intracellular portion of the human IgE receptor γ chain. This contains the signaling portion of the receptor and completes the gene for the chimeric T cell receptor. In order to enable better tracking of retroviral expression the gene for green fluorescent protein (GFP) was placed downstream of the chimeric receptor gene separated by an internal ribosomal entry site (IRES) to permit expression of the GFP from the same transcript as the chimeric receptor. Retroviruses with a single transcript containing a desired gene and GFP driven by an IRES have been made previously (7, 8). In our case the GFP gene was placed in the retroviral vector pSAM-EN (9) in place of the drug resistance gene and the chimeric receptor gene was inserted into the cloning site before the IRES. Lack of a second promoter avoids the problem of promoter interference. It also enables linked expression so that measuring the fluorescence of the GFP can

assess expression of the scFv. Transduced cells can be observed visually through a fluorescent microscope. This will permit the tracking of transduced cells in vivo (10, 11).

Better results were obtained with a stable packaging system (12, 13, 14, 15). A variety of different packaging cells were tried. The FLY series is based on the human cell line HT-1060 (16, 17). Packaging cell lines of these cells are available producing RD114, amphotropic, or GaLV envelope. Murine based packaging cell lines are available based on the 3T3 cell line. These cell lines are available to produce ecotropic, amphotropic or GaLV envelope proteins. These packaging cell lines were infected with virus from the Ping-Pong transfection (Figure 10). PG13 (18) providing the GaLV in a 3T3 based packaging cell line gave the highest expression of GFP. PA317 providing the amphotropic envelope worked almost as well (Table 2). The packaging cells can be sorted on the basis of the green fluorescence to give a population of cells producing virus. They can also be cloned by sorting cells into individual wells to give a virus producing line (Figure 11).

For optimal transduction of human peripheral blood lymphocytes with retroviruses the lymphocytes are activated. They are grown in RPMI with fetal calf serum (FCS) without IL-2 with plastic bound anti-CD3 and anti-CD28 antibodies for 48 hours. They are then infected with supernatant containing virus from the packaging cell line on plates coated with recombinant fibronectin fragments (Retrofectin™) (19). The infection is done in the presence of 50 U/ml of IL-2 for 5 hours. The cells are then grown for 24 hours in RPMI-FCS with 100 U/ml of IL-2 and the infection repeated for another 5 hours (Table 2). Low concentrations of IL-2 were used to prevent to propagation of natural killer cells. Higher concentrations are used for the production of lymphokine-activated killer (LAK) cells (20). LNCaP cell are deficient in their expression of MHC which would interfere with its ability to inhibit NK cell activity (21, 22).

The lymphocytes can be shown to express GFP by the green fluorescence and the scFv-CD28-Fcεγ receptor by staining the cells with a monoclonal antibody specific for the scFv. The stainings correlated in intensity verifying that determination of GFP expression is a suitable surrogate for the measurement of chimeric receptor expression (Figure 12).

Measuring the production of IL-2 in response to stimulation tested the functionality of the transduced receptor. Infected lymphocytes were starved for IL-2 and stimulated with plastic-bound TNP-fowl IgG. The lymphocytes responded to stimulation by producing IL-2 enabling lymphocyte survival. This survival was measured by an MTT assay (23). Different loadings of TNP were used (Figure 13).

These lymphocytes were able to kill tumor cell lines including LNCaP after they were TNP-ylated. The first cell line tried was the Burkitt tumor-derived, human B lymphoblastoid cell line, Raji which is resistant to NK killing although sensitive to killing by lymphokine-activated killer (LAK) cells. Efficient killing was obtained in only 4 hours at an effector to target ratios as low as 2:1 (Figure 14). Other B lymphoblastoid cell lines, such as the human Daudi and the Balb/C mouse A20, were also good targets for lymphocyte killing as was the prostate adenocarcinoma epithelial LNCaP (24) (Figure 15).

Based on the success with the anti-TNP scFv two other targeting molecules were placed into the same transfer vector in place of the scFv. These targeting molecules were directed towards the heregulin receptor, which is composed of HER2 and HER3 or HER4. One is a scFv targeting HER2 based on the N29 monoclonal antibody (25, 26). Heregulin itself has been used as the targeting molecule for toxins, viruses and cells (27, 28, 29, 30, 31, 32, 33). In our case we have a YOL epitope tag to facilitate the detection of chimeric receptor expression by a monoclonal antibody (34).Indeed, human PBL, activated with anti- CD28+CD3 antibodies and transduced by supernatants of retrovector

producing packaging cells, expressed the chimeric receptors and GFP (30-40% of the cells were stained, data not shown). HER1, HER2, and HER3 are consistently found in cell lines and tumor samples, but HER4 is not (35, 36). The LNCaP cell line contains relatively high levels of both HER2 and HER3 compared to other prostate tumors (32, 37). The NDF-directed and the N29-directed human lymphocytes exhibit higher killing of LNCaP than non-directed lymphocytes (Figures 15, 16). However high background killing is evident in these samples, especially at high effector to target ratios. This may be the result of NK killing of the LNCaP as it is deficient in MHC which blocks NK killing (21, 22). Chinese hamster ovary (CHO) cells and 32D, a mouse hematopoietic cell line (38, 39, 40) display lower background killing. These cells have been transfected with HER2 together with HER3 (41, 42). When these cells are used as targets, HER2/3 dependent killing is much clearer (Figures 16, 17). Sensitivity is correlated to the level of expression of HER2/3. The CHO cells with HER2 and HER3 contain 60,000 NDF receptors with an affinity of 2 nM (41). The 32D cells with HER2 and HER3 express about 12,000 NDF receptors (42). LNCaP has about 15% the amount of HER2 that SK-BR3 has or 18-fold over-expression (32, 43).

Lentiviral based

HIV-based vectors have been shown to transduce $CD34^+$ cells which have not been pre-stimulated (44). This is consistent with the ability of lentiviral vectors having the ability to transduce non-dividing cells (45). The lentiviral packaging system we are using is also transient based (46). The envelope gene is the VSV G protein (47). The packaging plasmid provides gag, pol and tat. The transfer vector uses the HIV-1 promoter to make viral RNA. It also includes a woodchuck hepatitis virus post-transcriptional regulatory element to enhance expression of the transgene (48). During reverse transcription this promoter is inactivated and an internal PGK or CMV promoter is used

(49). The insert containing anti-TNP scFv-CD28-IgEγ with an IRES-GFP was inserted into this vector and it is awaiting transfection into 293T cells to make virus to infect lymphocytes.

Database analysis of Prostate Gene Expression

At first glance determination of cell specificity of prostate tumor mRNA would be difficult because of the many different cell types in the prostate and the enormous number of different RNA species produced. However two facts simplify the problem. Prostate tissue contains many different cell types however 95% of prostate tumors are derived from the epithelial population. The LNCaP, PC-3, and DU145 prostate tumor cell lines are of epithelial origin. Each cell expresses between 10,000 to 30,000 different genes consisting of 100,000 to 1,000,000 mRNA molecules (50, 51, 52, 53, 54). However abundant (3%) and intermediate abundance (0.1%) classes of mRNA are more useful than the rare class (<0.004%). Rare transcripts are more often housekeeping genes whereas tissue specific genes are more often expressed at high levels. Higher levels of expression are more useful for targets. The abundant and intermediate class of transcripts (500 different species) can be covered to a 90% probability of seeing each sequence once by 5,000 sequences. For example the Prostate Expression Database currently lists 55,000 sequences (55). Partial sequencing of randomly sequencing cDNA clones generate expressed sequence tags (56, 57). Analysis of these sequences can indicate organ specificity of expression, both quantitative and qualitative (58, 59). The number of ESTs that represent the same gene is a rough indication of the expression level of the gene (60). The unexpected expression of the T-cell receptor γ chain transcript in prostate was found this way (61). Sequences that are specifically expressed in prostate have been found this way (62).

We have used this database to determine the level of expression of various target genes such as the HER family of receptors. There are 46 sequences from HER3 (gb:humher3a) in the dbEST database which come from non-normalized libraries. Eight of these are from prostate and seven of these are from tumors. HER3 is relatively specific for prostate. For HER4 there are two sequences, neither of which are from prostate. For HER2 , of 1000 sequences examined 22 were from prostate, 16 of which were from normal tissue. For the EGF receptor there are 105 ESTs with only 5 from prostate. For PSMA there are 27 sequences 9 of which are from prostate. Prostate stem cell antigen (GB:AF043498) is found 46 times, 15 of those in the prostate. Prostase, a prostate specific protease (63), is represented 7 times, 6 from prostate.

The UNIGENE system sorts the EST sequence information from the dbEST collection into clusters (64). If all of the sequences in the cluster are derived from prostate tissue sources, it is possible that the gene is specific for prostate. The more genes in a cluster then the greater is the probability that the gene is specific for prostate and not just a statistical fluctuation. In this way new targets can be found on prostate cells. One such possibility is Hs.18866 which contains three members which are all from prostate. It codes for a dipeptidyl peptidase which is generally an extracellular enzyme. We identified it as an additional target for the anti-prostate T-body approach.

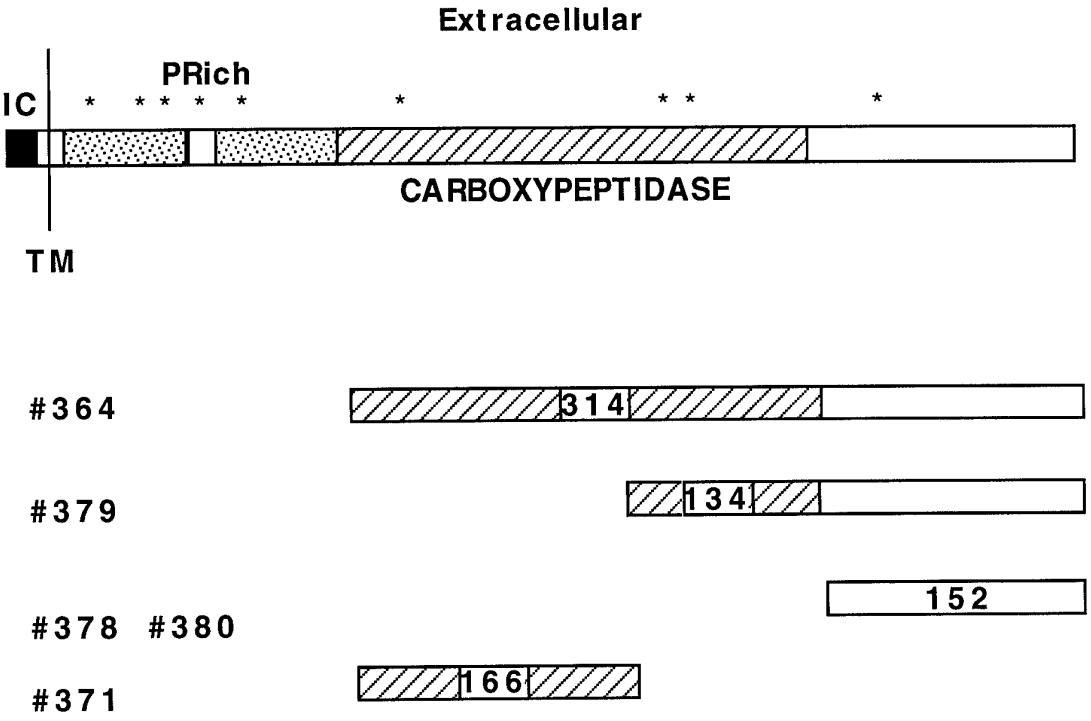

Figure 1-Schematic map of prostate specific membrane antigen.
IC is the amino terminal intracellular portion, TM is the transmembrane domain, Prich is the proline rich domain, and the carboxypeptidase domain. Asterisks mark the N-glycosylation sites. The extents of the different fragments cloned into different plasmids are indicated below the map. The size in amino acids is marked inside the bar.

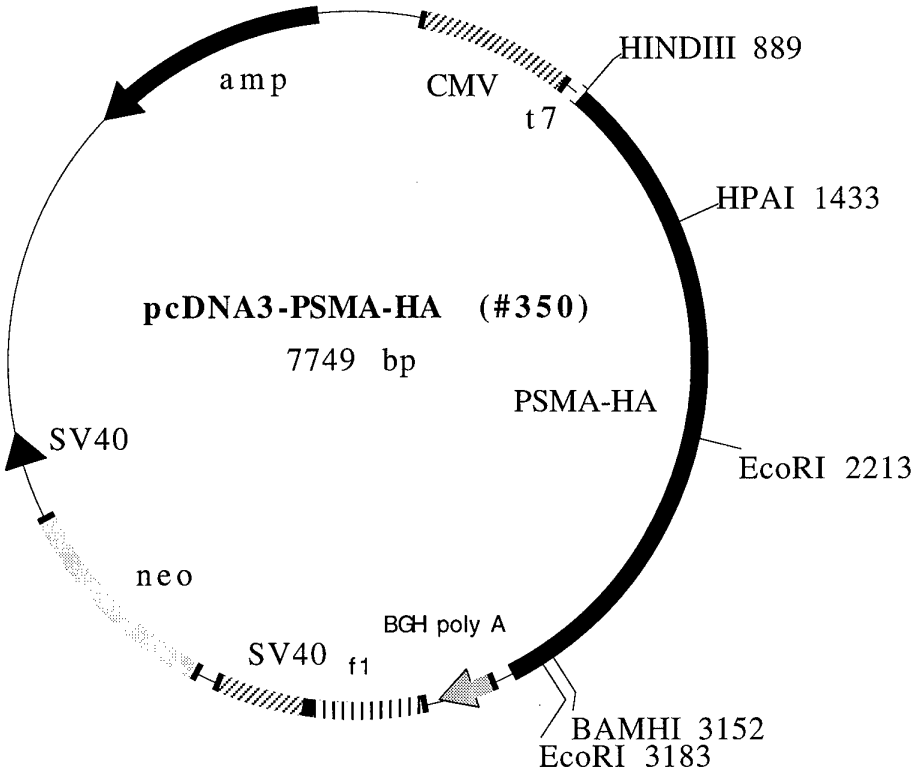

Figure 2- Schematic map of the plasmid for the expression of PSMA with a HA tag. A CMV IE promoter drives expression and the plasmid has a bacterial *neo* gene for selection in mammalian cells by G-418.

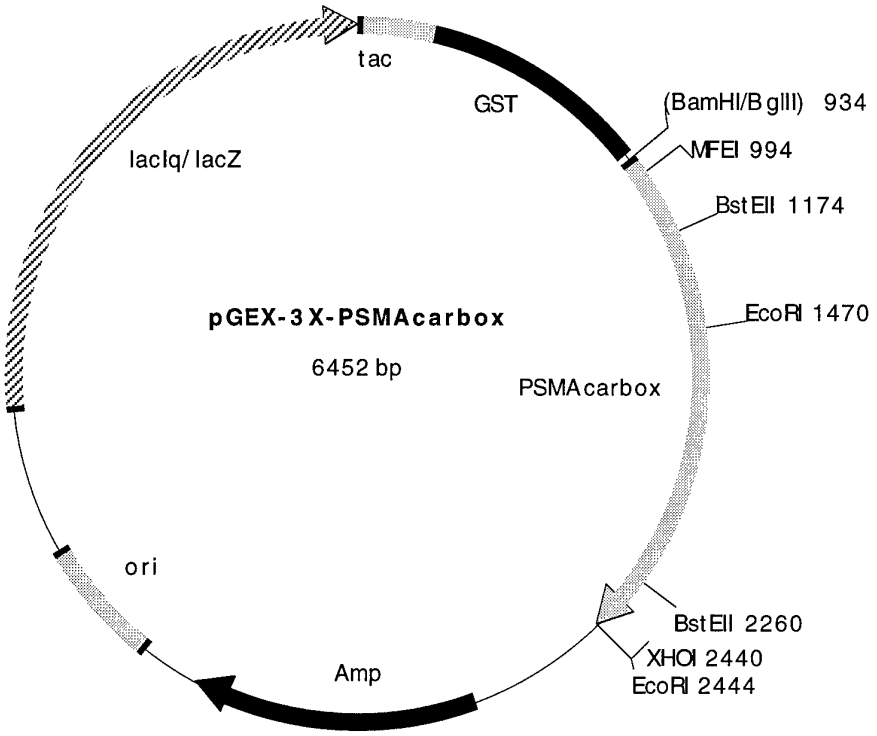

Figure 3- Schematic map of a pGEX plasmid for the bacterial expression of PSMA fragments. The gene lacI is the repressor for the hybrid trp-lac (tac) promoter. GST is glutathione-S-transferase. The piece is cloned between a BamH I site and an Eco I site.

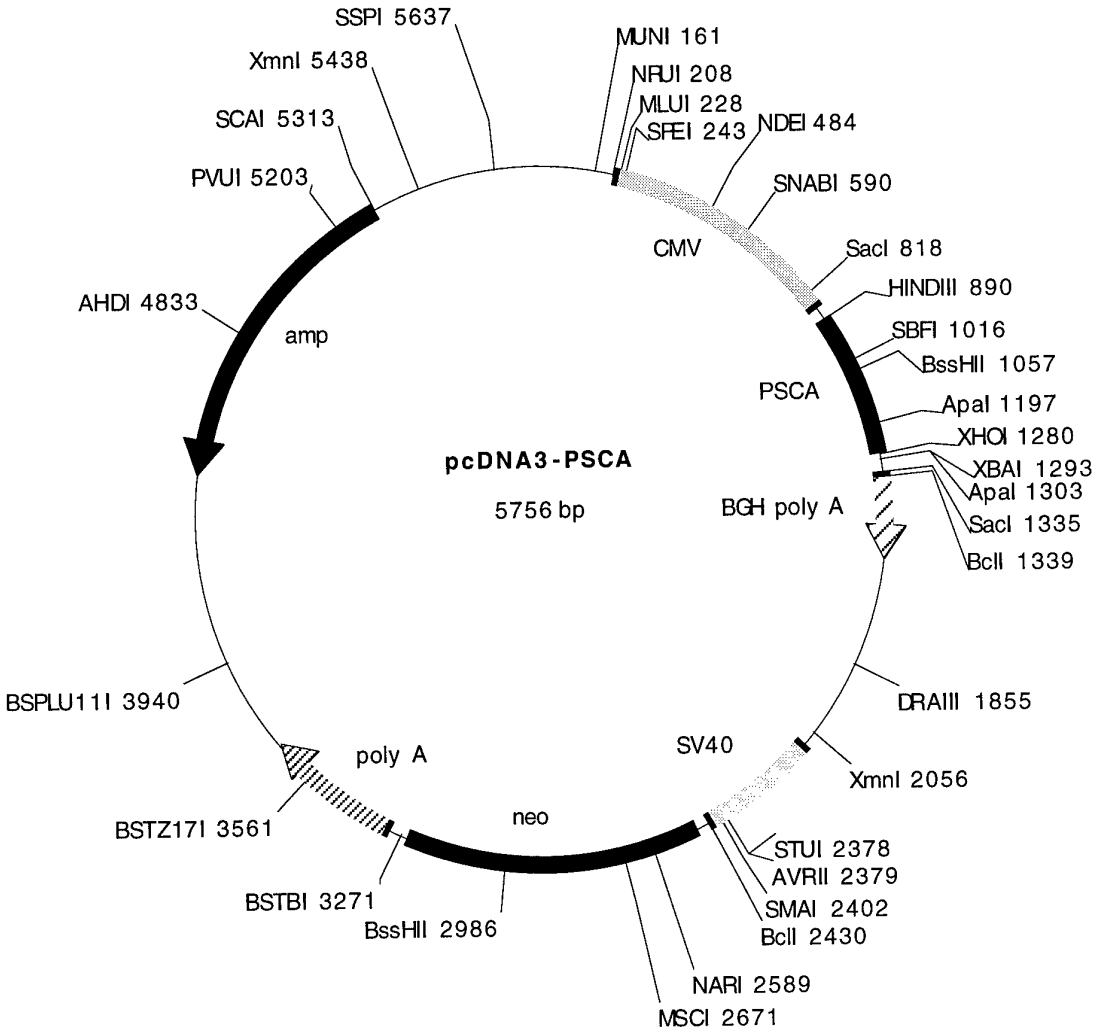

Figure 4- Schematic map of mammalian expression vector for PSCA. It is similar to the plasmid in figure 2. The cloning sites are the Hind III site at the 3' end and the Xho I site at the 5' end.

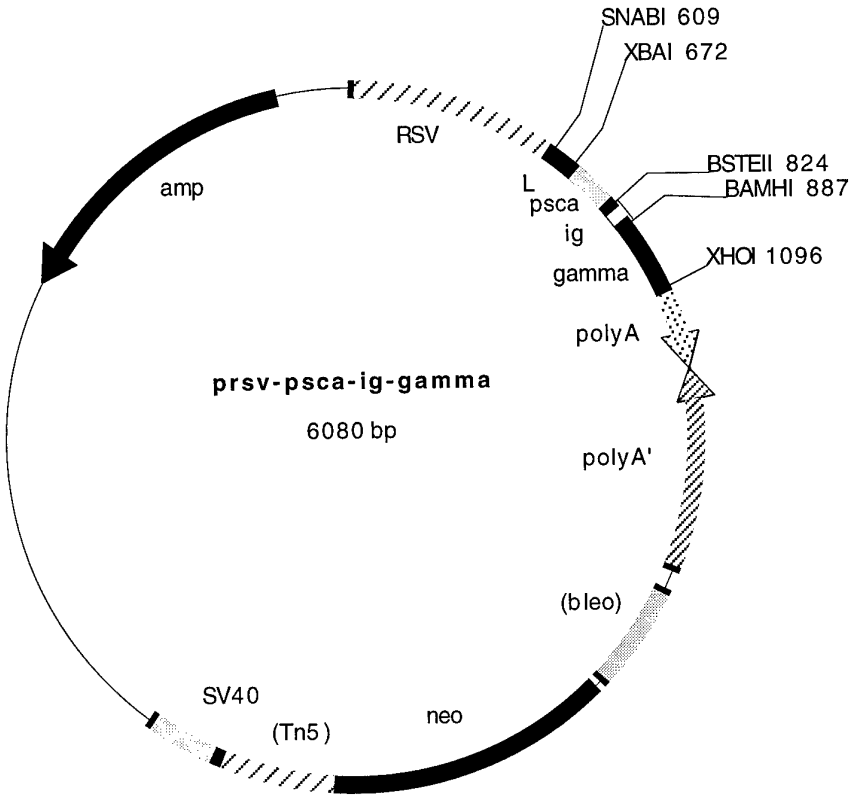

Figure 5- Schematic map of mammalian expression vector for surface expressed PSCA. A RSV LTR drives the expression and the plasmid has a bacterial *neo* gene for selection in mammalian cells by G-418. The mature PSCA protein is encoded by the sequence between the Xba I and the BstE II sites. The Ig portion serves as a spacer from the cell membrane and the gamma portion is from the Fce receptor gamma chain and provides a transmembrane segment as well as its intracellular portion.

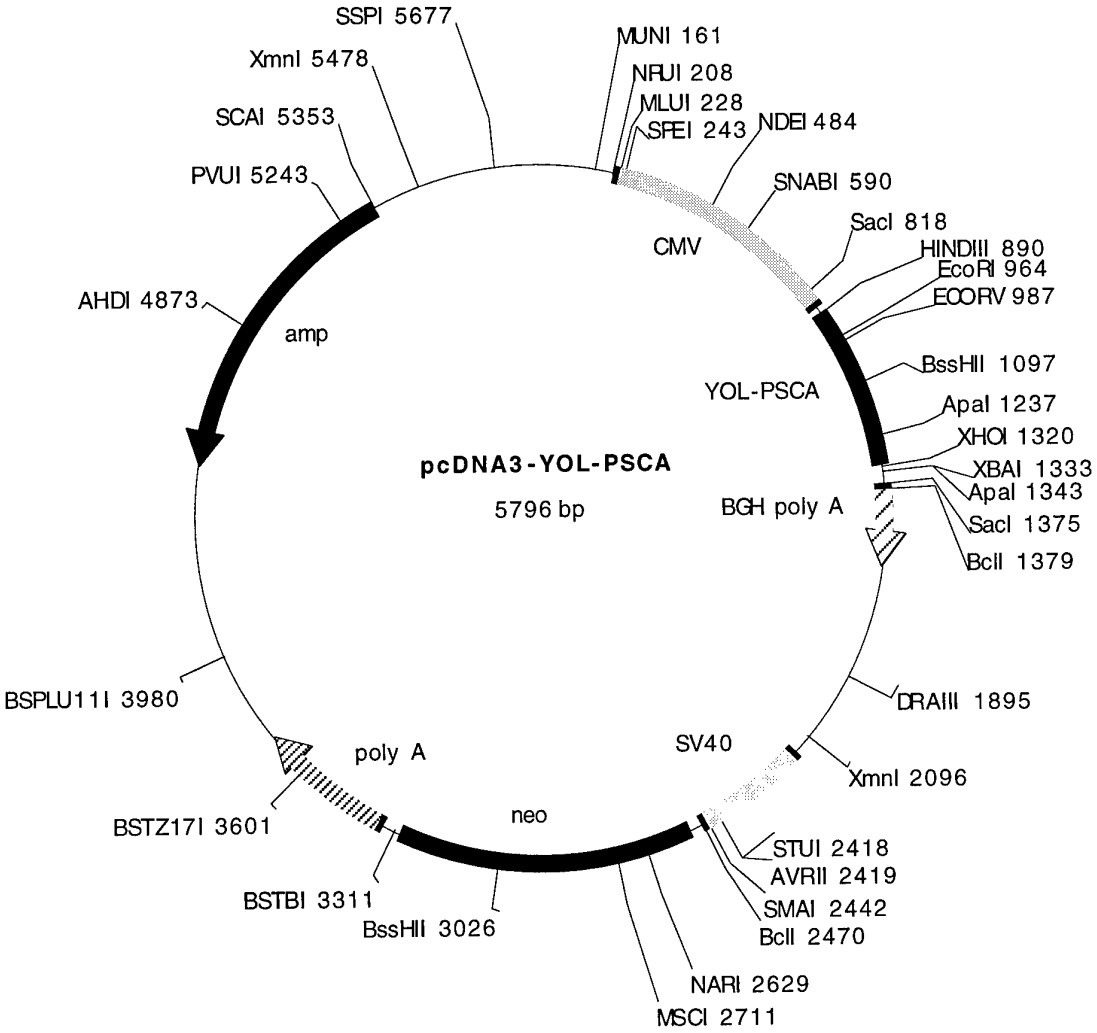

Figure 6- Schematic map of mammalian expression vector for surface expressed PSCA with a YOL tag for antibody detection. It is similar to the plasmid in figure 2.

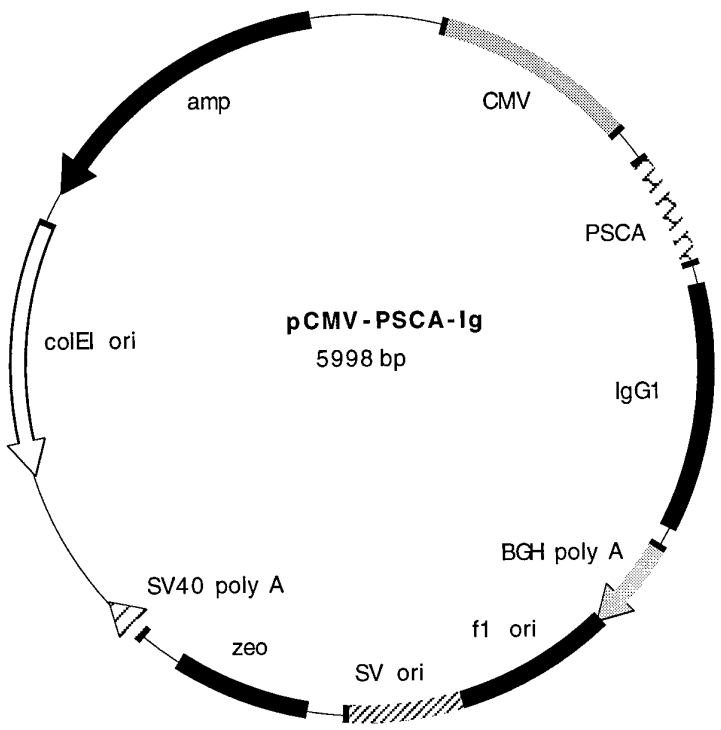

Figure 7- Schematic map of mammalian expression vector for the secreted PSCA-Ig fusion. The plasmid contains the *zeo* gene for selection with bleomycin derivatives.

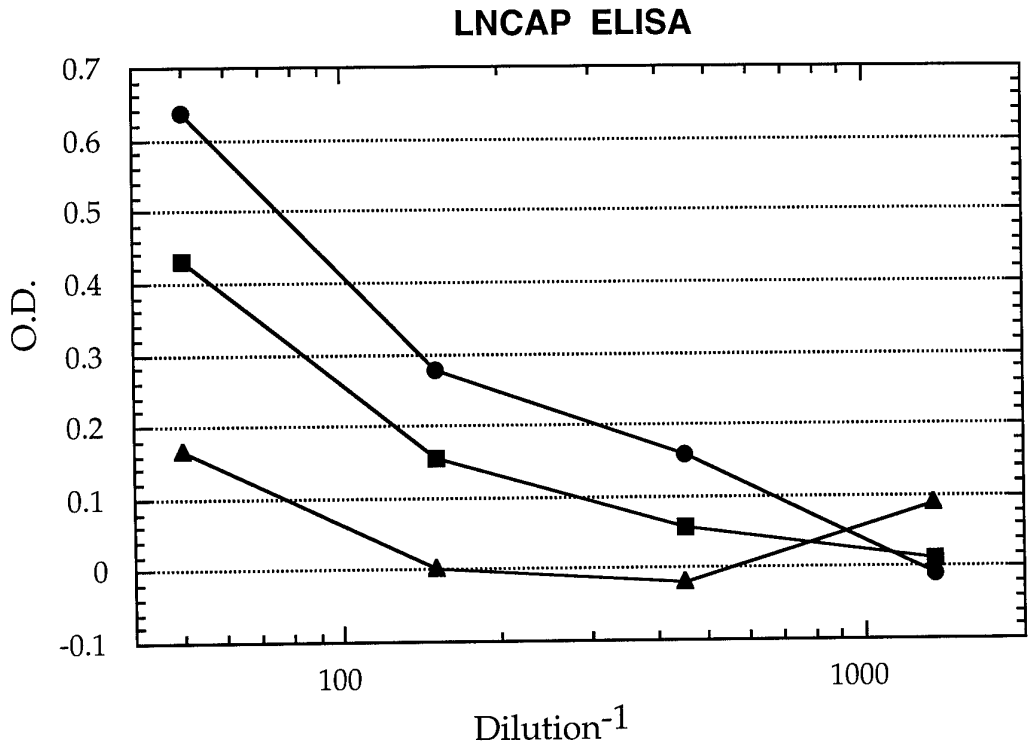

Figure 8-Testing immunized mouse sera on LNCaP cells with a whole cell ELISA. Circles are using sera PSMAII3, squares are from PSCA5, and triangles are from normal mouse sera. Values obtained using PBS have been subtracted.

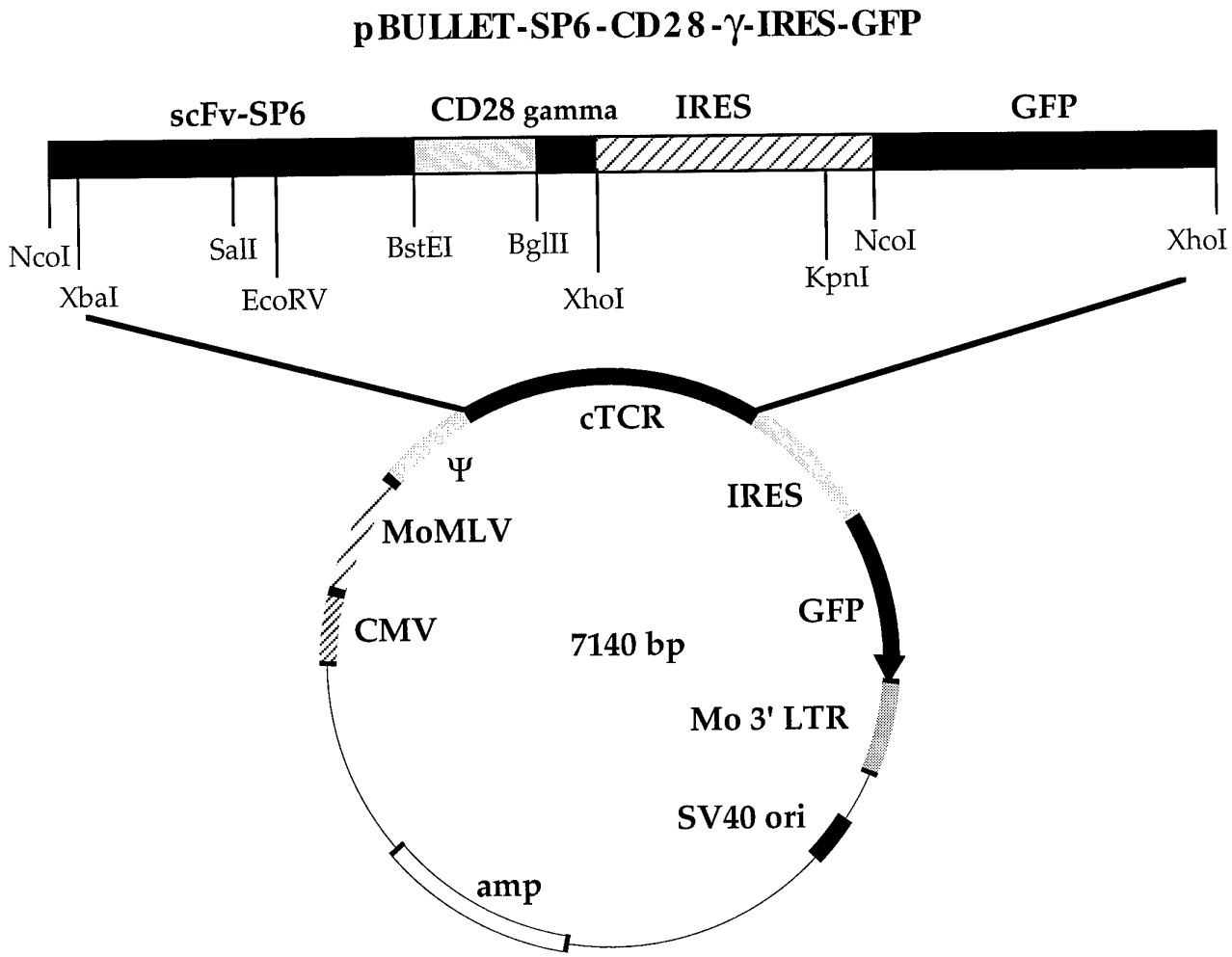

Figure 9- Schematic map of the retroviral transfer vector.

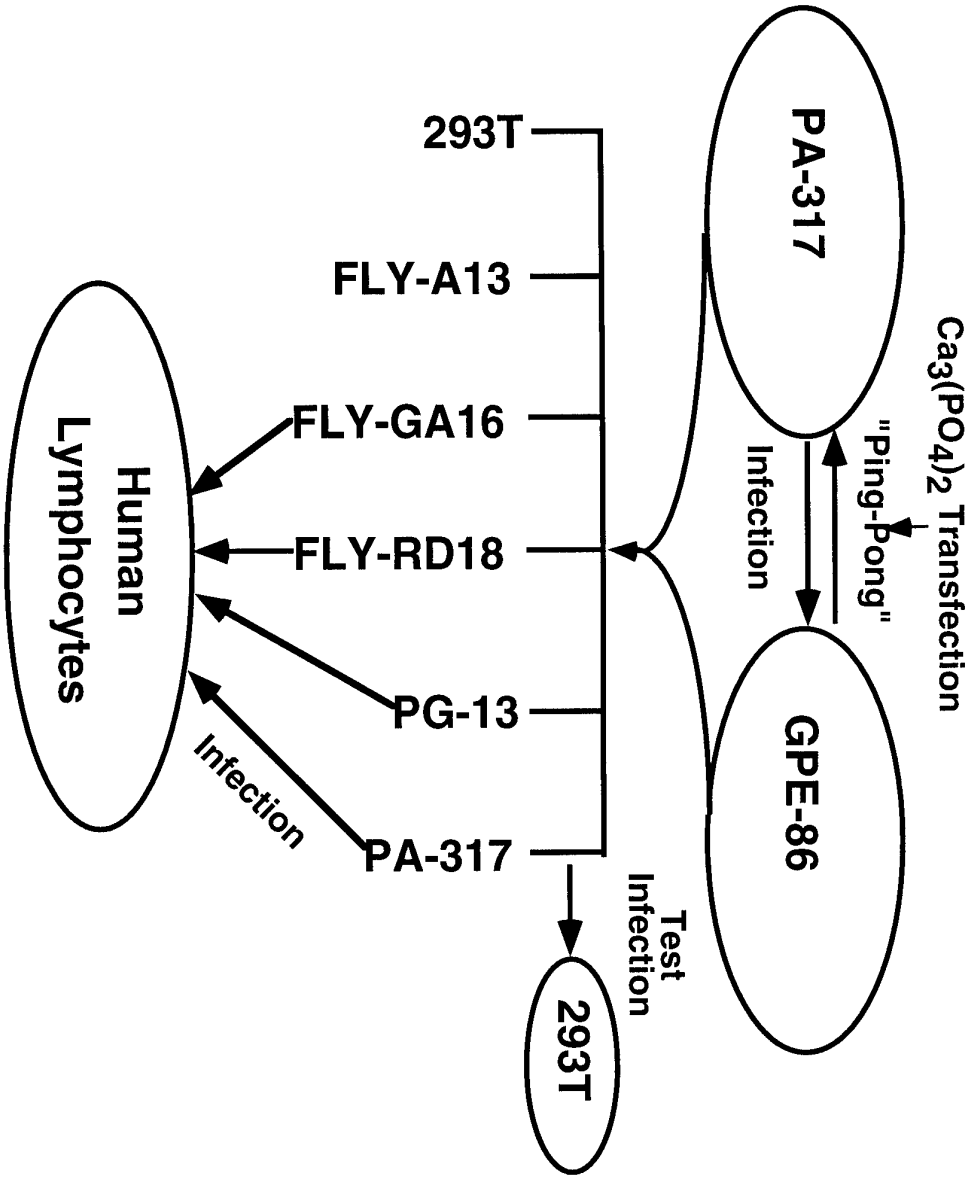

Figure 10- Schematic diagram of the experiment testing the ability of a variety of cells to produce virus.

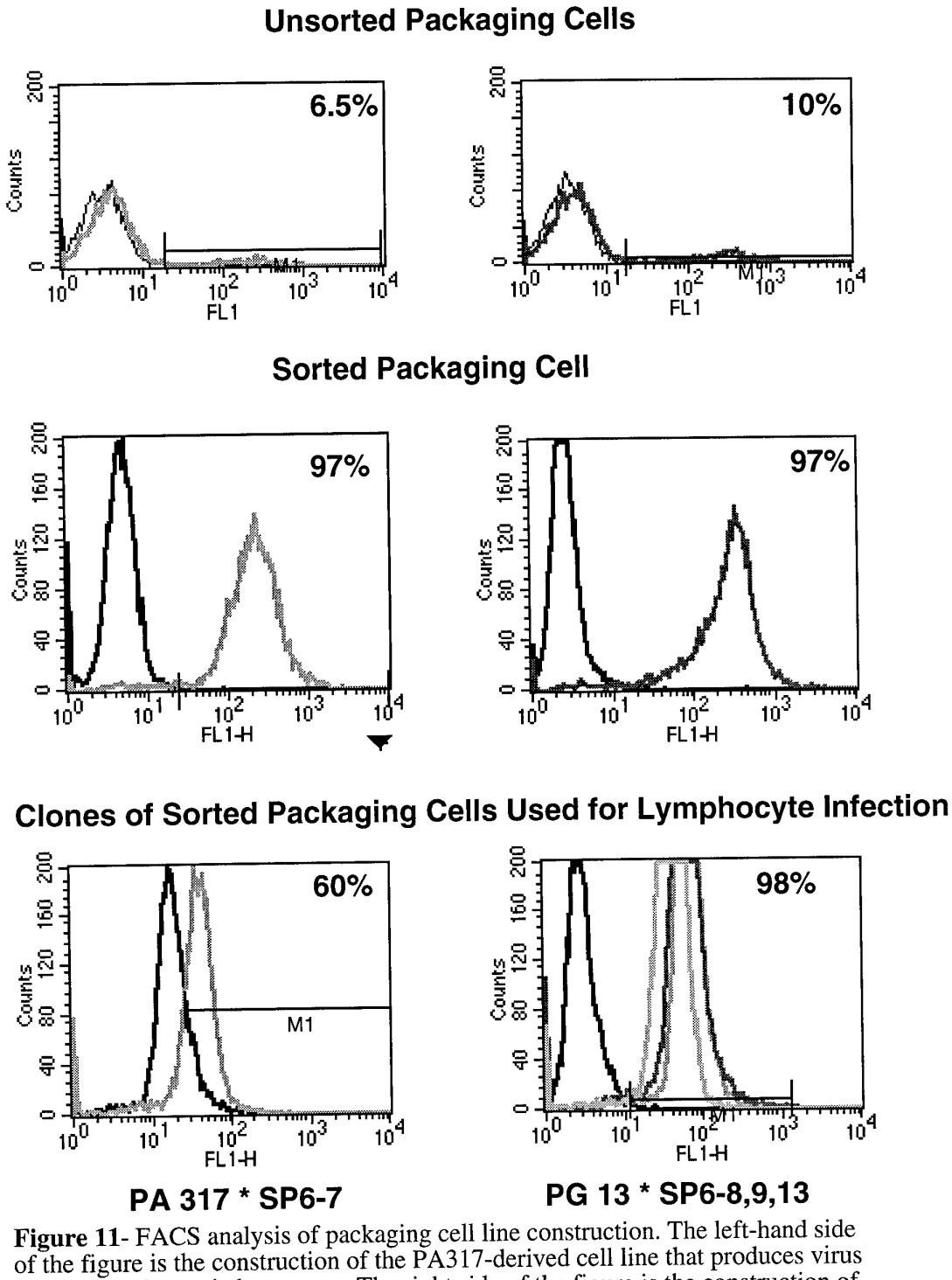

Figure 11- FACS analysis of packaging cell line construction. The left-hand side of the figure is the construction of the PA317-derived cell line that produces virus with an amphotropic host range. The right side of the figure is the construction of the PG13-derived cell line that produces virus with the gibbon ape leukemia virus envelope. The top figures are the results after the initial infection from the 'Ping-Pong' produced virus. For the infection of PA317, 6.5% of the cells expressed GFP and for the infection of PG13 10% produced GFP. The initial infected cells were sorted for fluorescence giving a population that was 97% positive for GFP in both infections. Clones from these infections were expanded and the ones from PG13 were very positive and the ones from PA317 were less so.

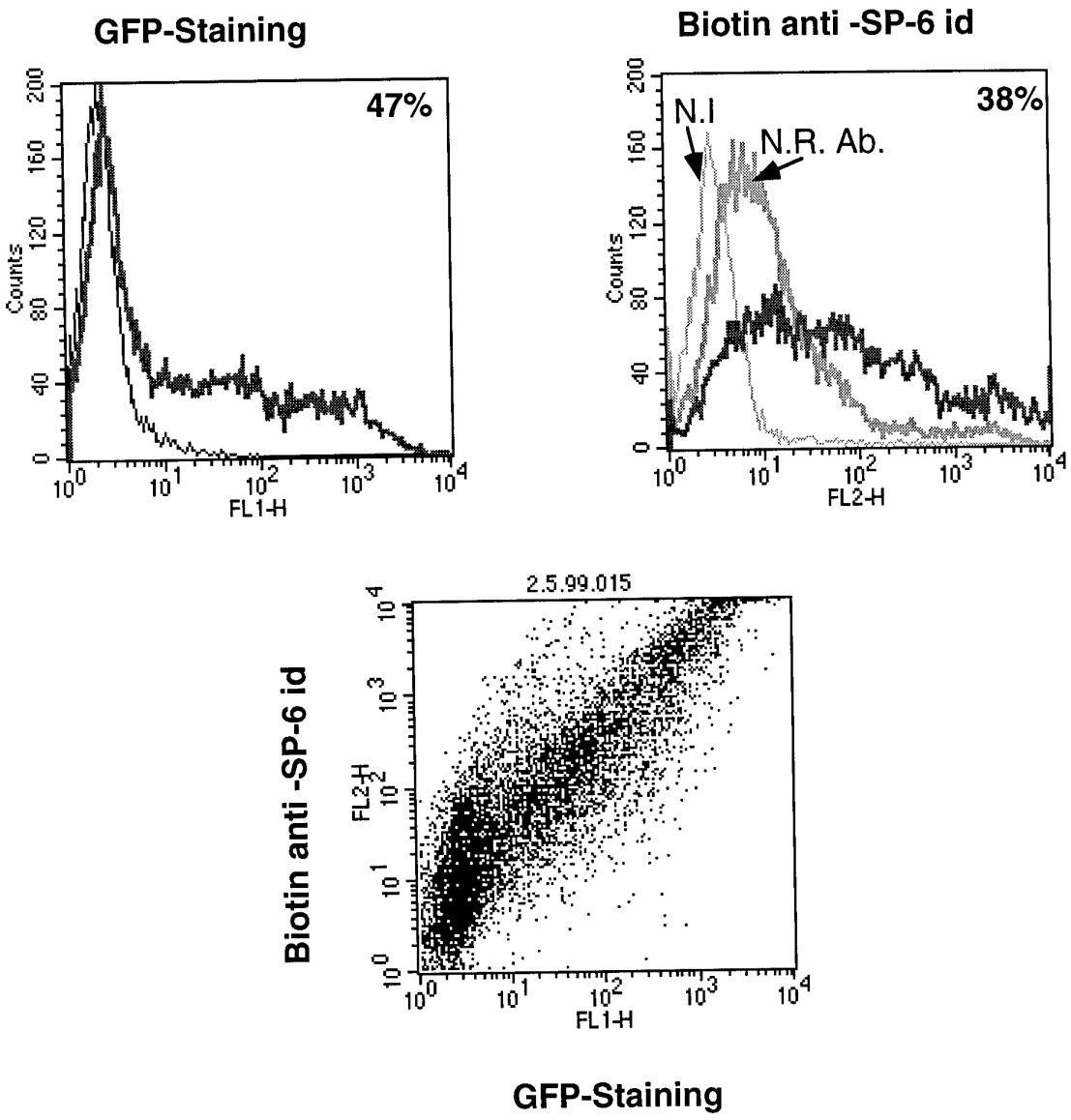

Figure 12- FACS analysis of infected human lymphocytes. The top left hand panel shows the transduction of the GFP gene by measuring fluorescence and the top right hand shows the transduction of the chimeric receptor by measuring the amount of receptor with biotinylated monoclonal antibody specific for the scFv. The receptor was stained with phycoerythrin-conjugated streptavidin. There are two negative controls. One is non-infected lymphocytes (N.I.) and the other is stained with a non-relevant antibody instead of the anti-scFv. The bottom panel shows the FACS measurement of both green GFP and red phycoerythrin at the same time to measure simultaneous expression of the GFP and the chimeric receptor.

Survival of Transduced Human Lymphocytes on Immobilized TNP-FγG

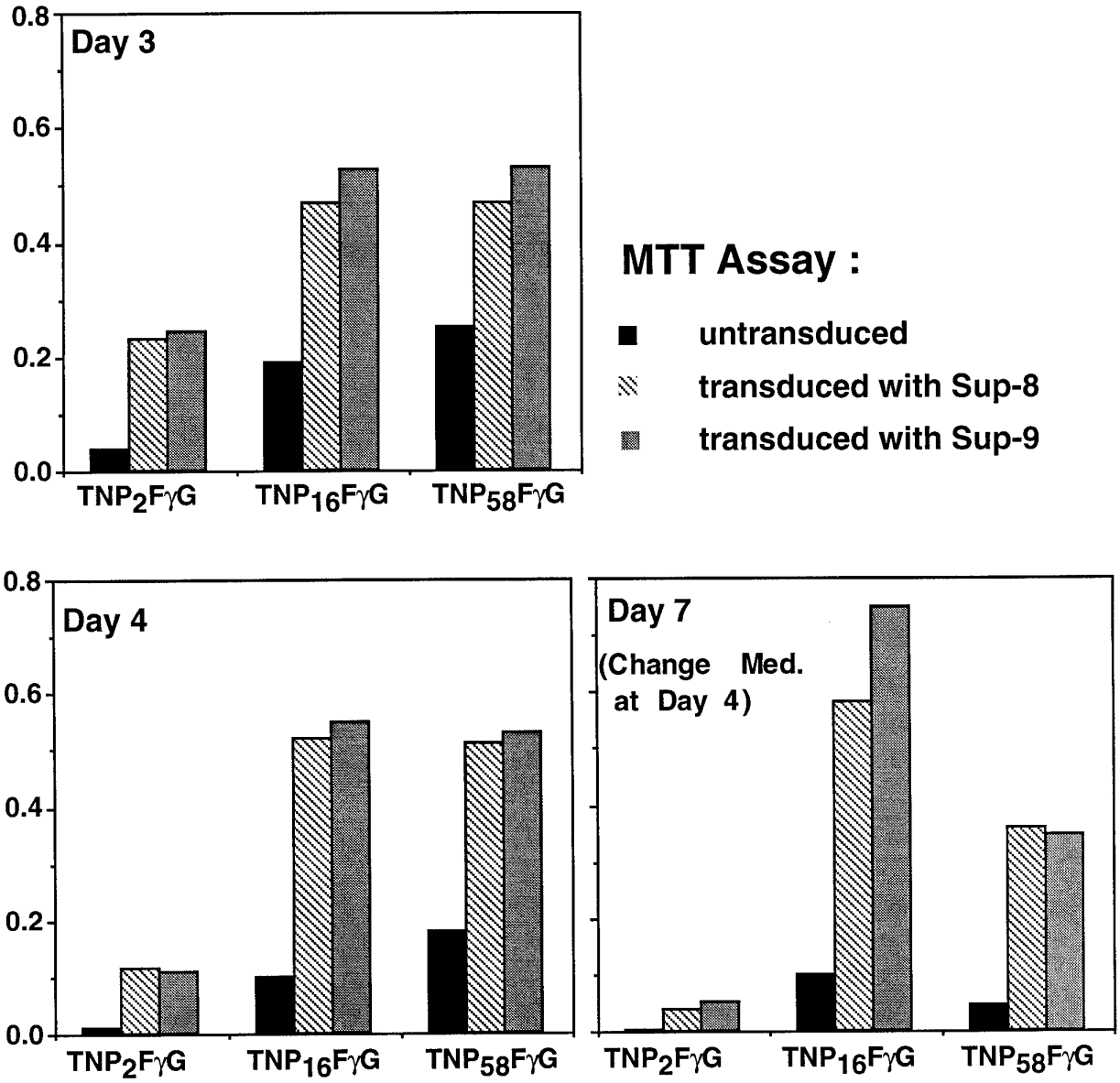

Figure 13- Infected human lymphocytes were grown on fowl immunoglobulin gamma globulin containing different loadings of TNP. The number of live cells was measured by an MTT assay.

Killing of Raji Cells by Human Lymphocytes Expressing TNP-Specific Chimeric Receptor

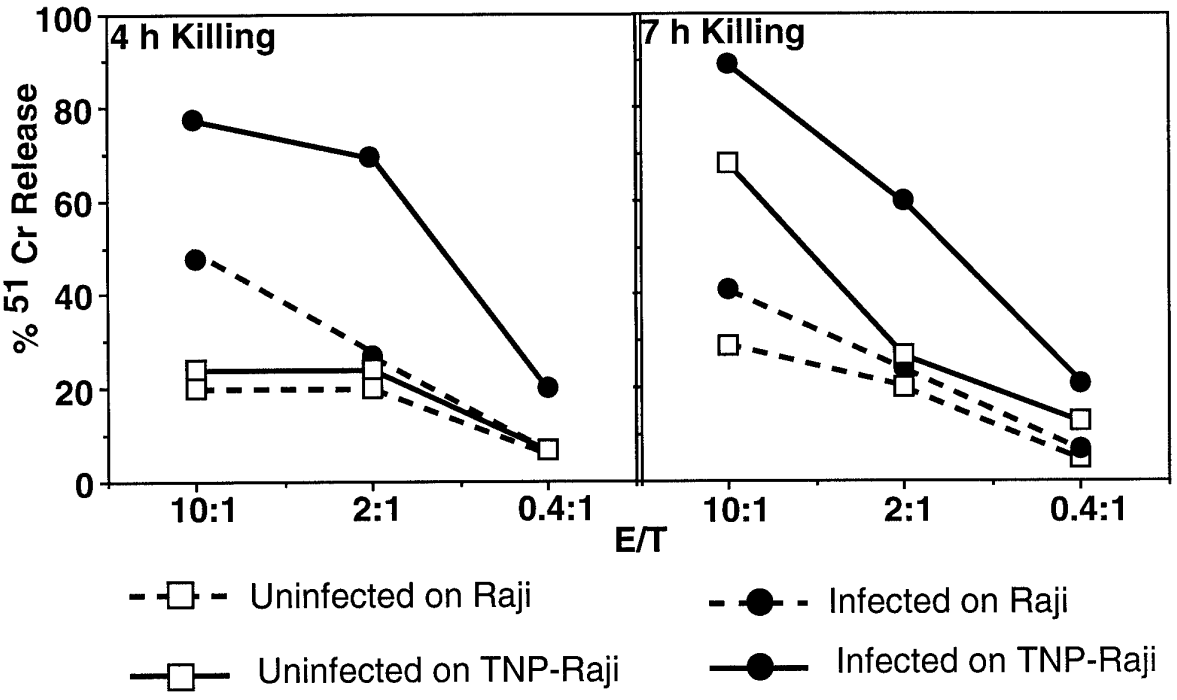

Figure 14- Killing of Raji target cells with human lymphocytes transduced with anti-TNP scFv receptor. The panel on the left measures killing by release of 51Cr after 4 hours and the one on the right after 7 hours. Dotted lines used unmodified Raji as the target and solid lines used TNP-ylated Raji as a target. Open squares are the results from uninfected human lymphocytes and filled circles use infected lymphocytes. Effector cell to target cell ratios varied from 0.4 to 10.

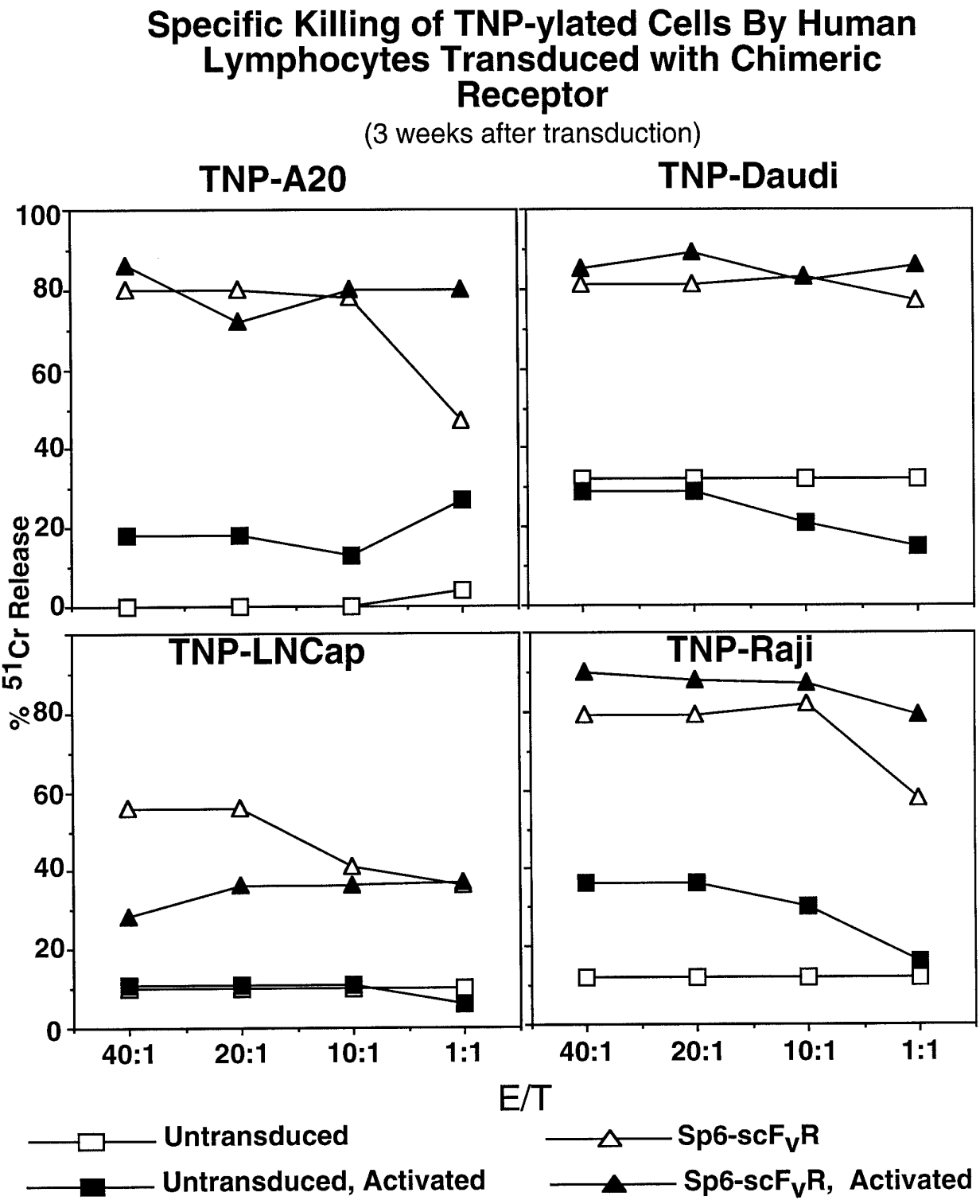

Figure 15- Specific killing of TNP-ylated target cells by human lymphocytes bearing anti-TNP scFv chimeric receptor. Squares are uninfected lymphocytes and triangles are infected lymphocytes. Solid symbols represent killing by activated human lymphocytes.

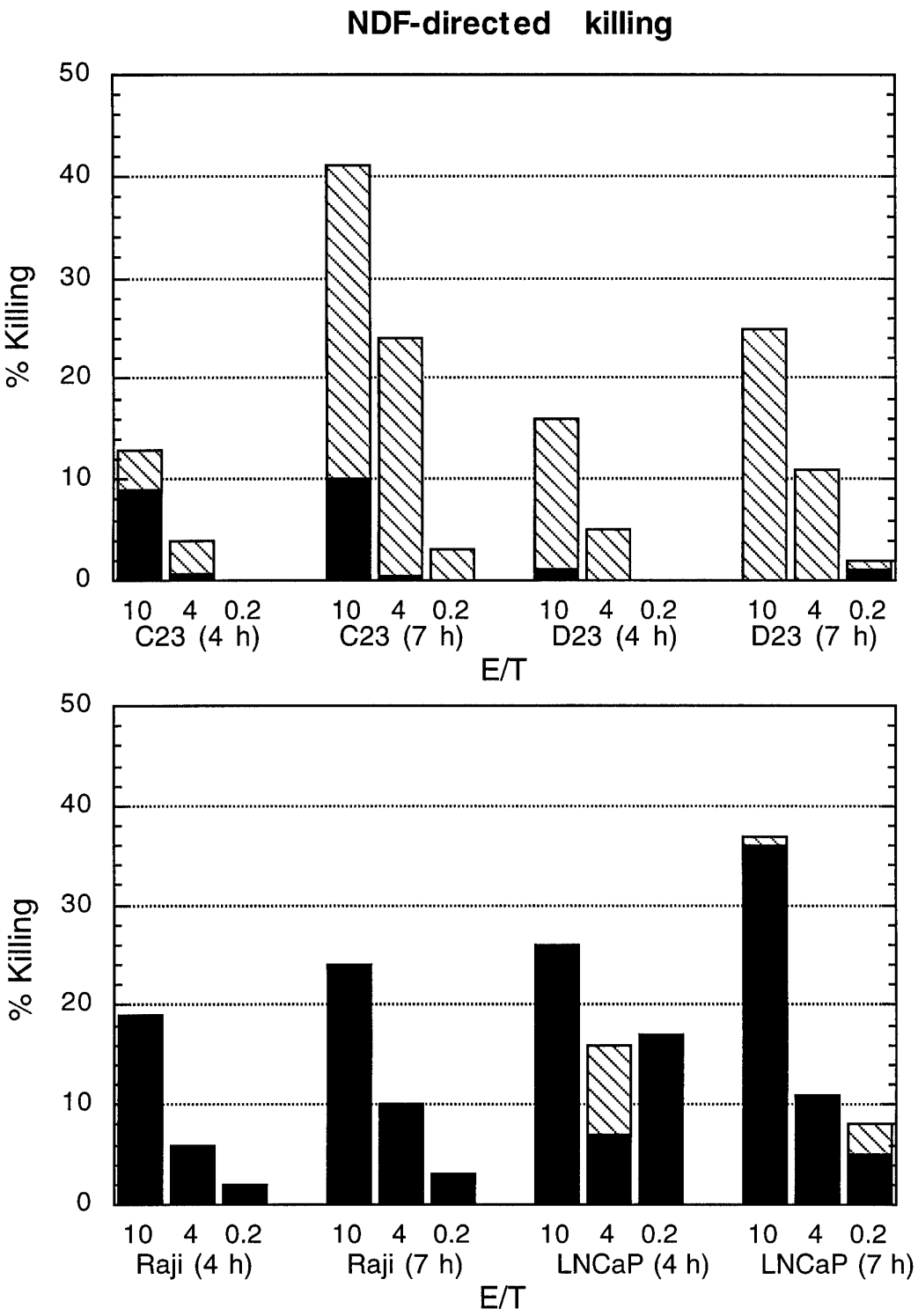

Figure 16- NDF-directed killing by transduced human PBL. PBL were transduced with a chimeric receptor bearing NDF. Killing is measured by the release of 51Cr. C23 is CHO transfected with HER2 and HER3. D23 is 32D transfected with HER2 and HER3. The solid part of the bar is the amount of killing done by uninfected lymphocytes whereas the striped bar is that done by transduced lymphocytes. The results for 2 different time points for each experiment are shown, 4 hours (4 h) and 7 hours (7 h).

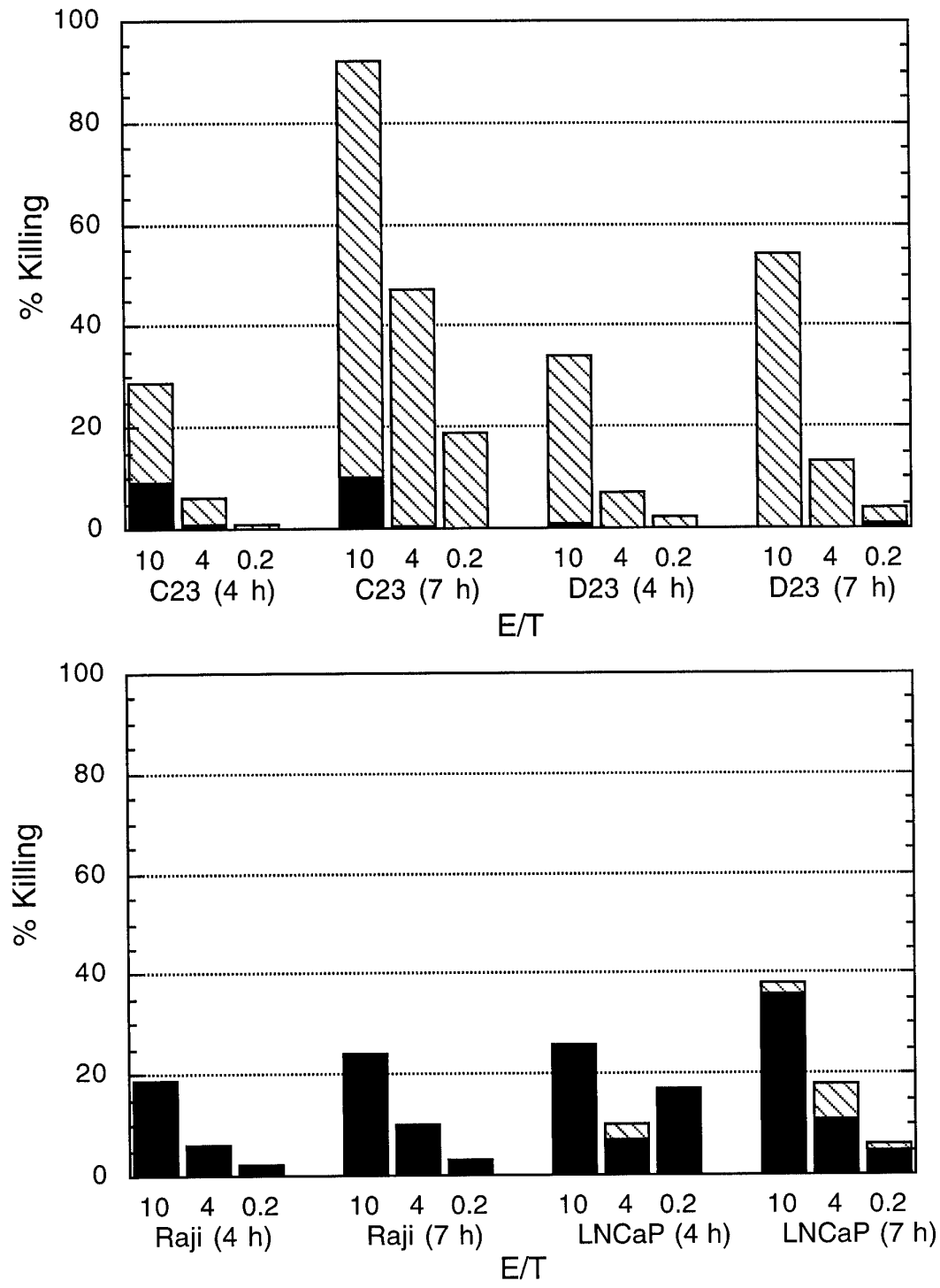

Figure 17- N29-directed killing by transduced human PBL. PBL were transduced with a chimeric receptor bearing an anti-HER2 scFv. The rest is as described for Figure 16.

Prostate progress report primers

Cloning PSMA

#26641 5' GCGAAGCTTGCGCCGAGATGTGGAATCTCCTTCACG

#26642 5' CTTGAATTCTCCTCTGCCCACTCAGTAGAACCAAGAAG

#26643 5' GGAGAATTCAAGACTCCTTCAAGAGCGTGGCGTGGCTTA

#26644 5' CGCGGATCCGCTACTTCACTCAAAGTCTCTGCAGCTGCCTGC

Expression of PSMA in pGEX

#29076 GGGATCCATTATGCTGTAGTTTTAAGAAAGTATGCTGACAAAATCTAC

#27910 GCGAATTCGCTCGAGTGGCTACTTCACTCAAAGTCTCTGC

#27909 5'

GCAGATCTCCGGATCCGCAAATGAATATGCTTATAGGCGTGGAATTG

Cloning of PSCA

#26586 5' CGCAAGCTTAGGCAGTGACCATGAAGGCTGTGCTGC

#26587 5' CCGCTCGAGCCTATAGCTGGCCGGGTCCCCAGAGC

Mammalian expression of PSCA

#27190 5' GCTCTAGACTGCAGCCAGGCACTGCCCTGCT

#27191 5' GCGGTGACCACGGCATGGGCCCCGCTGGCGTTG

#27237 5'

GCGAATTCTCCGAGGCCCGTGAGGATATCCTGCAGCCAGGCACTGCCCTG

#27186 GCGAATTCACCCTCCTCGGCCAAGCCTGCCATCA

Construction of PSCA-Ig

#27456 5' GCGGATCCGCAGCAGCCGGCTGCAGGGCATGGGC

Bacterial expression of PSCA

#28254 5' CGCGGATCCCCGATATCCTGCAGCCAGGCACTGCCCTGCT

#27748 5' GCGAATTCGCAGCCGGCTGCAGGGCATGGGCCCCGC

Table 2.

Infection of Different Packaging Cells

% positive clones			
	none	GFP	GFP-SP6
293T	0	0	0
FLYA13	0	0	0
FLYGA16	1.5	6	10
FLYRD18	1.5	7	12
PG13	0.5	7.5	9.5
PA317	0	3	0

Packaging Cells were infected twice by supernatant obtained following the "Ping-Pong" transfection. GFP Expression was monitored 24h latter.

Table 3.

GFP Expression in Human PBL Infected with Supernatants of Different Packaging Cells

Packaging Cells	48h		5 days	
	A	B	A	B
PA-317.7	<1	1	30	10
PG-13.8	10	40	30	50
PG-13.9	30	30	30	40
PG-13.13	1	15	40	50
FLY-GA16.1	0	0	5	1-5
FLY-RD18.4	0	0	>1	0

A : Infection according to K. E. Pollok et al.
J. of Virology, 72; 4882, 1998.

B : Infection according to M. Weijtens et al.
Gene Therapy, 5; 1195, 1998

Numbers are % infected cells as measured by fluorescence

Key Research Accomplishments

- Bacterial expression of PSMA and PSCA
- Mammalian Expression of PSCA-Ig
- Human T lymphocytes transduced with chimeric receptor genes kill a variety of targets in a specific and effective way

Reportable Outcomes

- Plasmid for bacterial expression of PSMA

- Plasmid for bacterial expression of PSCA

- Plasmid for expression of PSCA-Ig

- Preparation of PSMA protein fragments

- Preparation of PSCA

- Preparation of PSCA-Ig

- Mice immunized with PSMA

- Mice immunized with PSCA

- Development of bi-cistronic retrovector producing chimeric receptor and GFP

- Packaging cell line to produce retrovectors transducing anti-TNP chimeric receptors

- Packaging cell line to produce retrovectors transducing anti-HER2 chimeric receptors

- Packaging cell line to produce retrovectors transducing NDF specific chimeric receptors

-Post-doctoral fellow Zheng Zhu trained on this project

Conclusions:

In order to make antibodies to prostate tumor antigens, these proteins have been produced and found immunogenic in mice.

A retroviral transduction system has been set up to enable the transfer of functional chimeric receptor genes into human T cells

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